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Mitochondrial Effects of Estrogen are Mediated by ERα in Brain Endothelial Cells

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Abstract

Mitochondrial reactive oxygen species (ROS) and endothelial dysfunction are key contributors to cerebrovascular pathophysiology. Previously, we found that 17β-estradiol profoundly impacts mitochondrial function in cerebral blood vessels, enhancing efficiency of energy production and suppressing mitochondrial oxidative stress. To determine if estrogen specifically affects endothelial mitochondria through receptor mechanisms, we used cultured human brain microvascular endothelial cells (HBMEC). 17β-Estradiol treatment for 24 hours increased mitochondrial cytochrome c protein and mRNA; use of silencing RNA for estrogen receptors (ER) showed that this effect involved ERα, but not ERβ. Mitochondrial ROS were determined by measuring the activity of aconitase, an enzyme with an iron-sulfur center inactivated by mitochondrial superoxide. 17β-Estradiol increased mitochondrial aconitase activity in HBMEC, indicating a reduction in ROS. Direct measurement of mitochondrial superoxide with MitoSOX Red showed that 17β-estradiol, but not 17α-estradiol, significantly decreased mitochondrial superoxide production, an effect blocked by the ER antagonist, ICI-182,780. Selective ER agonists demonstrated that the decrease in mitochondrial superoxide was mediated by ERα, not ERβ. The selective estrogen receptor modulators, raloxifene and 4-hydroxy-tamoxifen, differentially affected mitochondrial superoxide production, with raloxifene acting as an agonist but 4-hydroxy-tamoxifen acting as an estrogen antagonist. Changes in superoxide by 17β-estradiol could not be explained by changes in manganese superoxide dismutase. Instead, ERα-mediated decreases in mitochondrial ROS may depend on the concomitant increase in mitochondrial cytochrome c, previously shown to act as an antioxidant. Mitochondrial protective effects of estrogen in cerebral endothelium may contribute to sex differences in the occurrence of stroke and other age-related neurodegenerative diseases.

Introduction

Effects of estrogen on mitochondria may be an important mechanism underlying the ability of this hormone to protect against cardiovascular disease and increase lifespan (Duckles et al., 2006). Mitochondria play a fundamental role in key cellular functions including oxidative phosphorylation, oxidative stress, and apoptosis (Wallace, 2005; Madamanchi and Runge, 2007). As a major by-product of energy production, reactive oxygen species (ROS) are generated in the mitochondrial matrix, and these affect mitochondrial enzymes, lipids, and DNA. In particular, chronic ROS exposure has been suggested to increase mitochondrial DNA (mtDNA) mutations that accumulate with age; mitochondrial dysfunction appears to be a major contributor to age-related diseases (Wallace, 2005; Madamanchi and Runge, 2007).

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Recent results indicate that estrogen suppresses mitochondrial ROS production (Stirone et al., 2005b; Duckles et al., 2006; Pedram et al., 2006b; Razmara et al., 2007). Interestingly, by this mechanism estrogen would be protective but would not be expected to reverse preexisting damage. Such a mechanism could explain the long-term positive benefits of ovary retention at the time of hysterectomy (Parker et al., 2005) and might also help explain the lack of effect of estrogen in recent hormone replacement therapy clinical trials, where subjects were 10 years or more past menopause (Harman et al., 2004).

Estrogen is believed to play a critical role in protecting females against vascular disease because the incidence of vascular disease-related events, such as stroke, rises dramatically in women following menopause (Mensah et al., 2005). In addition, a large body of evidence from animal studies strongly supports a vascular protective role of estrogen (Mensah et al., 2005). Estrogen is a multifaceted hormone with beneficial effects on vascular health through genomic as well as rapid, non-genomic pathways (Duckles et al., 2006; Krause et al., 2006). We recently demonstrated that estrogen modulates mitochondrial function in cerebral blood vessels (Stirone et al., 2005b), suggesting a novel mechanism for vascular protection. Specifically, estrogen increased cerebrovascular expression of several mitochondrial respiratory chain proteins, including cytochrome c and complex IV subunits. This correlated with estrogen-induced increases in the activities of the rate-limiting enzymes cytochrome c oxidase and citrate synthase. Estrogen also increased protein levels of the important mitochondrial-specific antioxidant enzyme, MnSOD, and decreased mitochondrial H_2O_2 production, indicating increased efficiency of mitochondrial function (Stirone et al., 2005b). Others have shown that estrogen increases MnSOD expression in blood vessels (Strehlow et al., 2003) and stabilizes the mitochondrial membrane, thus maintaining ATP production (Moor et al., 2004). Estrogen increases cerebrovascular levels of nuclear respiratory factor-1 (Stirone et al., 2005b), suggesting the hormone acts through key transcriptional coactivators responsible for regulating mitochondrial function and oxidative metabolism.

Endothelial dysfunction and reactive oxygen species (ROS) production are major contributors to vascular disease, hypertension, and atherosclerosis (Madamanchi and Runge, 2007). Cerebrovascular endothelial cells have particularly high metabolic requirements in order to maintain the blood-brain barrier (Abbott et al., 2006), thus mitochondrial ROS production may be especially relevant to cerebrovascular endothelial dysfunction and may contribute to the pathophysiology of stroke and other age-related diseases of the brain (Abbott et al., 2006).

Because of the potential importance of the ability of estrogen to suppress mitochondrial ROS in cerebrovascular endothelium, in this study we investigate the impact of physiological levels of the primary endogenous estrogen, 17β-estradiol, on mitochondrial superoxide production in cultured human brain microvascular endothelial cells, a critical component of the blood-brain barrier. We measured mitochondrial ROS using several complementary techniques and investigated possible mechanisms by which mitochondrial oxidative stress may be modulated by estrogen. In particular we investigated the role of estrogen receptors, ERα and ERβ, in mitochondrial effects of estrogen and compared 17β-estradiol with two clinically relevant selective estrogen receptor modulators (SERMs), raloxifene and 4-hydroxy-tamoxifen, to see if these drugs also influence endothelial mitochondrial ROS.

Methods

Cultured Endothelial Cells

Primary human brain microvascular endothelial cells (HBMEC) were purchased from Applied Cell Biology Research Institute through Cell Systems (Kirkland, WA) and grown at 37°C in endothelial growth media (CSC Complete Medium; Cell Systems), with 5% CO_2 and addition of antibiotics and an antifungal agent (100 μg/ml streptomycin, 100 U/ml penicillin and 2.5

μg/ml fungizone) according to the supplier's recommendation. Cells were grown in flasks coated with Cell Systems attachment factor and used for experiments between passages 6 to 10. The endothelial identity of the cells was confirmed with von Willebrand factor immunocytochemistry. Hormone experiments were initiated at 70–80% cell confluence when complete media was replaced with hormone-free medium, incomplete CSC medium (without phenol red or serum) supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin, and 2.5 μg/ml fungizone and 1–2% fetal bovine serum previously charcoal-stripped of steroid hormones.

HBMEC were treated with either 10 nM 17β-estradiol (encapsulated in 2-hydroxy-propyl-β-cyclodextrin; Sigma), 10 nM 17α-estradiol (Sigma), 10 nM 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT, Tocris, Ellisville, MI), 10 nM 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN, Tocris), 100 nM 4-hydroxy-tamoxifen (Sigma), 100 nM raloxifene (a gift from Eli Lilly, Indianapolis, IN), or equivalent concentration of 2-hydroxy-propyl-β-cyclodextrin alone (vehicle control), and the cells were incubated for 24 h at 37°C in 95% O_2-5% CO_2. Some HBMEC were pre-treated with the estrogen receptor antagonist ICI-182,780 (1 μM; Tocris; Ellisville, MI) or 100 nM 4-hydroxy-tamoxifen for 1 h and then drug exposure continued during the 17β-estradiol or vehicle treatment. After 24 h treatment, cells were collected for mitochondrial isolation, RNA isolation, and biochemical assays.

Immunocytochemistry

HBMEC were seeded in 6-well dishes containing 22-mm glass coverslips coated with Cell Systems attachment factor and immunocytochemistry performed for von Willebrand factor, and subunit I of complex IV and measurement of MitoSOX Red fluorescence. Co-localization studies were performed for MitoSOX Red and subunit I of complex IV. Cells were incubated with 2.5 μM MitoSOX Red (Molecular Probes, Eugene, OR) for 45 min at 37°C, then fixed with 3.7% paraformaldehyde for 15 min at 37°C, permeabilized with 0.2% Triton X-100 (Sigma; St. Louis, MO) for 10 min, and blocked for 1 h in 1% BSA-0.05% Triton X-100 prior to antibody incubation. After overnight incubation at 4°C with either anti-von Willebrand factor (1:200 dilution, Santa Cruz Biotech, Santa Cruz, CA) or anti-subunit I of complex IV (1:200 dilution; Molecular Probes), the cells were washed with PBS and then incubated with 1 μg/ml Alexa Fluor 488 (Molecular Probes) fluorescent secondary for 2 h. After washes in PBS, cover slips were then partially dried, and placed on glass slides with mounting medium (Vector Laboratories, Burlingame, CA). Images were obtained using a Carl Zeiss Axiovert 200M fluorescent microscope equipped with appropriate filters. Appropriate controls, such as secondary antibody alone, were used to confirm lack of non-specific staining.

Mitochondria Isolation

Mitochondria were isolated from HBMEC using a mitochondrial isolation kit (MITO-ISO1, Sigma), following manufacturer's guidelines with additional centrifugations to obtain an enriched, purified mitochondrial fraction (Stirone et al., 2005b). Briefly, freshly collected HBMEC were homogenized in an extraction buffer (50 mM HEPES, pH 7.5, 1 M mannitol, 350 mM sucrose, and 5 mM EGTA). The HBMEC homogenate was centrifuged twice at 600g for 5 min at 4°C to separate out the nuclear pellet. Then, the supernatant was centrifuged at 11,000g for 10 min at 4°C for separation of the mitochondrial pellet and cytosolic fractions. The pellet was resuspended and spun again at 11,000g for 10 min to obtain an enriched mitochondrial pellet. Western immunoblot analysis of porin protein was used to confirm the identity of the mitochondrial fraction. Western blots for histone H1, a nuclear marker, were used to show the absence of nuclear contamination.

Enzyme Activities of Aconitase/Fumarase and Aconitase Reactivation

Mitochondrial aconitase is sensitive to inactivation by superoxide due to the susceptibility of its iron-sulfur core to oxidation; however, fumarase is unaffected. Thus, the activity ratio of aconitase to fumarase was calculated as an indicator of mitochondrial ROS production. Aconitase and fumarase enzyme activities were measured in mitochondria from vehicle or drug-treated HBMEC using spectrophotometric rate determination (Gardner et al., 1994). Freshly isolated HBMEC mitochondria samples were freeze-thawed for three cycles, followed by centrifugation at 16,000g for 5 minutes. For aconitase activity measurement, aliquots of the mitochondrial fraction were incubated in a reaction buffer that contained 154 mM Tris, 5 mM sodium citrate, 0.6 mM $MgCl_2$, and 0.2 mM $NADP^+$ at 25°C. Specific activity of mitochondrial aconitase was measured by monitoring the conversion of citrate to α-ketoglutarate over time at 340 nm at 25°C using the coupled reduction of $NADP^+$ to NADPH by 2 units/ml isocitrate dehydrogenase. Aconitase reactivation was achieved by incubation of mitochondrial samples for 5 min with the reducing reagents, 2 mM dithiothreitol and ferrous ammonium sulfate at 0.2 mM. Then the enzyme activity assay was repeated as described above. One milliunit of aconitase activity was defined as the amount catalyzing the formation of 1 nmol of isocitrate per min (Gardner et al., 1994), and values were normalized to protein content. The percent inactivation of aconitase was calculated as the difference between mitochondrial aconitase activity before and after reactivation, divided by the reactivated activity, and multiplied by 100.

Fumarase activity was measured by conversion of malate to fumarate. Aliquots of the mitochondrial fraction were incubated in a reaction buffer of 0.05 M potassium phosphate buffer, pH 7.4, and 0.05 M sodium L-malate. The specific activity of fumarase was subsequently determined by monitoring the absorbance over time at 240 nm as fumarate end-product accumulated. The ratio of the specific activities of aconitase to fumarase was then calculated and expressed relative to vehicle control.

Mitochondrial Superoxide Production

Intramitochondrial superoxide production was measured in live HBMEC treated with hormones and drugs using the MitoSOX Red reagent (Molecular Probes, Eugene, OR). This fluorogenic dye selectively enters mitochondria within living cells, where it is oxidized by superoxide anions. This oxidation reaction then emits a red fluorescence when bound to mitochondrial DNA, which is measured using a fluorescence spectrophotometer. After 24 h hormone or drug treatment, HBMEC were incubated with 2.5 μM MitoSOX for 10 min, followed by wash cycles with a warm 1X Hanks' balanced salt solution (Invitrogen, Carlsbad, CA) before 30 min fluorescence readings at wavelengths 510/580 nm (excitation/emission) using a Perkin Elmer Luminescence Spectrophotometer LS50B. After initial baseline stabilization, production of mitochondrial superoxide was induced by addition of the complex I substrates, sodium pyruvate and malic acid (2 mM each). The superoxide dismutase mimetic, Mn(III) tetrakis (4-benzoic acid) porphyrin (MnTBAP, 1 mM), was used to scavenge superoxide produced in the reaction to confirm that the MitoSOX signal reflects superoxide levels. Superoxide production was calculated as fluorescence/min/mg protein over a linear range and expressed relative to vehicle control.

Immunoblot Analysis

Endothelial cells and enriched mitochondrial pellets from HBMEC were resuspended at 4°C in cell lysis buffer (50 mM β-glycerophosphate, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DL-dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride, with 100 μM $NaVO_3$, 0.5% Triton X-100, 20 μM pepstatin, 20 μM leupeptin, and 0.1 U/ml aprotinin; all chemicals from Sigma) and assayed for protein concentration using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

Equal amounts of whole cell lysate or mitochondrial sample proteins, dissolved in Tris-glycine SDS sample buffer with β-mercaptoethanol, were loaded onto 8% or 16% Tris-glycine gels (Invitrogen; Carlsbad, CA) and separated by SDS-polyacrylamide gel electrophoresis. Molecular weights of protein bands were determined using broad-range molecular weight markers (Bio-Rad, Hercules, CA). Proteins were then transferred to a nitrocellulose membrane (Amersham Pharmacia, Piscataway, NJ), incubated overnight at 4°C in blocking buffer, and treated with primary antibodies of interest: MnSOD (Sigma); cytochrome c, ER-α (HC-20), ER-β (H-150), GAPDH, and histone H1 (Santa Cruz Biotechnology, Santa Cruz, CA); and porin (CalBiochem, San Diego, CA). To verify equal loading, GAPDH was used for whole cell samples, and porin was used for mitochondrial samples. After incubation with appropriate secondary antibodies, protein bands were detected by enhanced chemiluminescence with Hyperfilm (Amersham Pharmacia) and analyzed by densitometry using UN-SCAN-IT (Silk Scientific, Orem, UT).

Manganese Superoxide Dismutase Activity Assay

Manganese superoxide dismutase activity in HBMEC was measured using a SOD assay kit (Cayman Chemical; Ann Arbor, MI). Isolated HBMEC mitochondria samples were freeze-thawed for three cycles, then centrifuged at 16,000g for 5 minutes, and protein-normalized mitochondrial samples were used for the assay. Superoxide radicals generated by xanthine oxidase were detected using a tetrazolium salt with absorbance monitored at 450 nm. A standard curve produced by using known amounts of exogenous SOD to quench radicals generated by xanthine oxidase was used to compare to endogenous MnSOD activity in HBMEC mitochondria samples. Only mitochondrial MnSOD activity was detected since 1 mM potassium cyanide was used to inhibit other forms of SOD, including Cu/Zn-SOD and extracellular SOD. Using the generated standard curve, the MnSOD activity per amount of mitochondria protein was quantified, and then corrected relative to vehicle control.

Quantitative Real-Time PCR

RNA extraction was performed using Trizol reagent according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA), and purified after DNase RNAse free (Roche, Mannheim, Germany) treatment with RNeasy columns (Qiagen, Valencia, CA). First strand cDNA synthesis was then performed using the SuperScriptTM kit (Invitrogen), and quantitative real time-PCR (qRT-PCR) performed using the FastStart DNA Master SYBR Green kit in a Light Cycler (Roche Molecular Biochemicals). MnSOD and cytochrome c mRNA levels were normalized to an actin amplicon and the results compared to a standard curve. Oligonucleotide forward and reverse primers used for human MnSOD were (5′-ttggccaagggagatgttaca-3′, 5′-cgttagggctgaggtttgtcc-3′) and human cytochrome c (5′-gaagtgttcccagtgccaca-3′, 5′-gctattaagtctgccctttcttcc-3′). Relative amounts of transcripts were calculated on the basis of crossing point analysis. The results from three independent amplifications for each sample, analyzed in duplicate, were combined for statistical analysis.

RNA Interference

ERα and ERβ RNA interference (RNAi) construct sequences were generously provided by Dr E. Levin and have been previously validated and published (Pedram et al., 2006a). Constructs were ordered from Qiagen, with the ERα DNA target sequence as: aagcccaaatgtgttgtggcc, and the ERβ target sequence as: aaggtgggatacgaaaagacc. As a negative control, we used a random sequence RNAi construct with no homology to any known mammalian gene (Qiagen). HBMEC at 70–80% confluence were transfected with an RNAi construct, at 5 nM, using Oligofectamine reagent in OptiMEM reduced serum medium (Invitrogen), according to the manufacturer's protocol. Control cells were treated with the transfection vehicle alone. After

48 h transfection, cells were treated with 10 nM 17β-estradiol or vehicle (2-hydroxy-propyl-β-cyclodextrin) for 24 h, and then collected for RNA isolation and/or immunoblot analysis.

Statistical Analysis

All data values are given as mean ± SEM. Statistical differences were determined by Student's t-test, or where appropriate, one-way ANOVA with repeated measures. The latter approach was used due to variability in values among assays. Thus, every assay included a sample from each of the relevant experimental treatments, all run at the same time. ANOVA was followed by Newman-Keuls post-hoc analysis. In all cases, statistical significance was set at $P \leq 0.05$.

Results

Estrogen Increases Mitochondrial Cytochrome c via ERα

Cytochrome c is a critical mitochondrial carrier protein involved in energy production, apoptosis, and ROS production. In Figure 1, we show that 17β-estradiol treatment of HBMEC for 24 h at 10 nM significantly increases cytochrome c mRNA (Fig. 1A) and protein (Fig. 1B).

Immunoblot analysis of estrogen receptors showed that both the 66 kDa (Fig. 2A) and 45 kDa (Fig. 2B) forms of ERα (Stirone et al., 2003) and a 55 kDa band (Fig. 2C) corresponding to ERβ were present in HBMEC. In order to investigate which estrogen receptor mediates the influence of estrogen on mitochondrial cytochrome c, we used short interfering RNA constructs to reduce levels of either ERα or ERβ in HBMEC. Confirming the effectiveness of short interfering RNA constructs specific for either ERα or ERβ, each of these significantly reduced the respective receptor protein levels at 48 h post-transfection, whereas there was no effect on the other receptor subtype nor was there an effect of the negative control RNAi construct (Fig. 2).

We then measured the effect of 17β-estradiol on mitochondrial cytochrome c protein in cells pretreated with or without estrogen receptor RNAi targeting either ERα or ERβ (Fig. 3). As a control, we validated that neither RNAi construct alone, without 17β-estradiol, had any effect on cytochrome c protein levels (Fig. 3). Figure 3A and B show that a loss of ERα protein significantly inhibits the ability of 17β-estradiol to increase cytochrome c. In contrast, the loss of ERβ did not affect the estrogen-mediated increase in cytochrome c (Fig. 3C and D).

Estrogen Decreases Mitochondrial ROS and Increases Aconitase Activity

Mitochondrial aconitase activity is a functional indicator of mitochondrial levels of ROS, because the iron-sulfur core of aconitase is oxidized by superoxide, reducing enzyme activity. Mitochondrial ROS, however, does not affect fumarase activity. Therefore we utilized the activity ratio of mitochondrial aconitase to fumarase (A/F) as a functional indicator of ROS production in HBMEC (Fig. 4). Higher A/F ratios signify lower mitochondrial ROS production. As shown in Figure 4A, the ratio of mitochondrial aconitase to fumarase was significantly increased by 17β-estradiol treatment. To demonstrate that the effect of 17β-estradiol does not depend on alterations of aconitase enzyme levels, total aconitase activity was measured after reactivation by exposure to a reducing agent (Fig. 4B). After aconitase reactivation there was no difference in total reactivated aconitase activity among treatment groups, supporting the conclusion that estrogen did not alter enzyme protein levels. As a measure of the degree of aconitase inactivation in various groups, we calculated the percent difference in mitochondrial aconitase activity before and after reactivation. This value was 25 ±5% after treatment with 17β-estradiol and 67±3% for vehicle treatment, emphasizing the difference in mitochondrial aconitase activity after 17β-estradiol treatment and the ability of 17β-estradiol to lower mitochondrial ROS production in HBMEC.

Estrogen Suppresses Mitochondrial Superoxide Production

To directly measure mitochondrial superoxide production, we used the MitoSOX Red probe in HBMEC after various hormone and drug treatments. To confirm the expected mitochondrial localization of MitoSOX Red, immunocytochemistry was used (Fig. 5A–C). Figure 5A shows MitoSOX red fluorescence, while Fig. 5B shows immunofluorescence corresponding to subunit I of complex IV, a protein encoded by mtDNA and restricted to the mitochondria. The merged image (Fig. 5C) demonstrates that MitoSOX fluorescence within the cell co-localizes with immunofluorescence for subunit I of complex IV, confirming the mitochondrial localization of the MitoSOX probe.

Representative fluorescence intensity tracings in Figure 5D show HBMEC pre-treated with 17β-estradiol (10 nM) or vehicle. At first, the baseline mitochondrial superoxide levels were low, rising upon addition of the complex I substrates pyruvate and malate. For cells treated with 17β-estradiol, the slope of the fluorescence tracing is lower than that of vehicle-treated HBMEC. When the superoxide dismutase mimetic, MnTBAP, was added after the tracing reached a plateau, there was a sharp fall in fluorescence intensity reflecting superoxide scavenging by MnTBAP. This validates the use of the MitoSOX Red probe as a measurement of mitochondrial superoxide. Figure 5E shows values for the rate of superoxide production, relative to vehicle, corrected with respect to protein concentration in each sample. In 17β-estradiol-treated HBMEC, mitochondrial superoxide production was significantly suppressed compared to vehicle control. This effect was specific to 17β-estradiol, since the enantiomer, 17α-estradiol, did not alter superoxide production. Furthermore, the estrogen receptor antagonist, ICI-182,780, inhibited the ability of 17β-estradiol to decrease mitochondrial superoxide. Together, these data support the concept that 17β-estradiol acts via estrogen receptors to decrease mitochondrial superoxide production.

Effects of Selective ER Agonists and ER Modulators on Mitochondrial Superoxide Production

We then tested selective ER agonists and ER modulators to further explore the nature of the estrogen receptor that mediates suppression of mitochondrial superoxide. As shown in Figure 6A, the ERα selective agonist PPT significantly decreased superoxide levels, while the ERβ selective agonist DPN had no effect. These effects were observed using 10 nM for each ER agonist, a concentration previously shown by others to selectively activate the respective estrogen receptor (Harrington et al., 2003). Figure 6B demonstrates that the selective estrogen receptor modulator, raloxifene (100 nM), also significantly decreased superoxide production. In contrast, 4-hydroxy-tamoxifen (100 nM), the active metabolite of the partial estrogen agonist, tamoxifen, had no effect on superoxide production. However, 4-hydroxy-tamoxifen did inhibit the suppressive effect of 17β-estradiol on mitochondrial superoxide levels. This observation suggests that tamoxifen, through its active metabolite, has no agonist activity but acts like an antagonist. These experiments with selective ER agonists support the conclusion that ERα is responsible for the action of estrogen to suppress superoxide production, while raloxifene, but not 4-hydroxy-tamoxifen, is capable of the same activity.

Effect of Estrogen on Manganese Superoxide Dismutase (MnSOD)

Previously we have shown that chronic estrogen treatment of female rats increases MnSOD protein levels in mitochondria isolated from cerebral blood vessels (Stirone et al., 2005b) as well as enzyme activity in brain mitochondria (Razmara et al., 2007). Thus, to explore the molecular mechanism of the effects of estrogen on mitochondrial oxidative stress, we investigated this important mitochondrial antioxidant protein. As shown in Figure 7A, after 24 h treatment of HBMEC with 10 nM 17β-estradiol, MnSOD RNA levels were unchanged. Figure 7B demonstrates that levels of MnSOD protein were also unaffected by exposure to 17β-estradiol. Finally, 17β-estradiol did not alter activity of MnSOD (Fig. 7C). Thus, we did

not see any significant change in mRNA, protein, or activity of MnSOD in HBMEC treated with 17β-estradiol for 24 h. Therefore, after 24 h 17β-estradiol exposure, suppression of superoxide production cannot be accounted for by a change in MnSOD function.

Discussion

The most striking finding of this paper is the profound suppressive effect of estrogen on mitochondrial superoxide production in cultured human brain endothelial cells. Physiological levels of estrogen significantly decreased mitochondrial superoxide production and increased mitochondrial aconitase activity, a functional indicator of mitochondrial ROS levels. These effects were receptor-mediated through ERα. Considering its important role in superoxide metabolism, we also measured MnSOD mRNA, protein, and activity; these were unaltered after estrogen treatment. As an alternative explanation for suppression of superoxide production, we found that through ERα estrogen upregulates cytochrome c mRNA and protein. In light of the antioxidant role of cytochrome c through the electron leak pathway (Skulachev, 1998; Zhao et al., 2003; Zhao and Xu, 2004), an increase in mitochondrial cytochrome c may account for the suppressive effects of estrogen on mitochondrial ROS production.

Typically, oxygen-derived free radicals function as intracellular messengers in cell growth and proliferation (Droge, 2002). During cerebral ischemia and reperfusion injuries, however, excess ROS causes DNA damage, protein oxidation, and lipid peroxidation (Madamanchi and Runge, 2007). The deleterious effects of ROS eventually lead to endothelial dysfunction, vascular smooth muscle cell proliferation, and destabilization of atherosclerotic plaques (Strehlow et al., 2003).

Mitochondrial oxidative phosphorylation is a major source of free radical by-products (Madamanchi and Runge, 2007) that produce long-lasting changes in mitochondrial function; especially in highly metabolically active tissues, such as endothelial cells of the blood-brain barrier (Nag, 2003). Mitochondrial ROS leads to oxidative damage to mtDNA, which lacks robust repair mechanisms, so accumulation of mtDNA mutations over the lifespan is considered to be a major contributor to age-related loss of function (Wallace, 2005). Disruption of cerebrovascular function, including the blood-brain barrier, is thought to contribute to the pathophysiology of a broad spectrum of age-related brain disorders, including Alzheimer's disease and stroke (Abbott et al., 2006). Thus, if estrogen reduces mitochondrial ROS production and delays accumulation of mtDNA mutations, estrogen would be expected to slow or delay the onset of age-related brain disorders.

Estrogen is a pleiotropic hormone with multiple sites and mechanisms for its profound neuroprotective effects (Singh et al., 2006); in particular the cerebral vasculature is a target for sex hormones (Krause et al., 2006). Since both sexes have endogenous estrogen, protective effects of estrogen may occur in both men and women (Roof and Hall, 2000). Specifically, in male rodents estrogen has been shown to attenuate stroke damage (Toung et al., 1998), reduce cerebrovascular inflammation (Razmara et al., 2005), and suppress brain mitochondrial oxidative stress (Razmara et al., 2007). These protective effects of estrogen have also been observed under pathological conditions in various neuronal cell types (Green and Simpkins, 2000) and endothelial cells (Razandi et al., 2000).

Here, we found that 10 nM 17β-estradiol depresses brain endothelial mitochondrial ROS production using two complementary techniques: mitochondrial superoxide with MitoSOX Red and mitochondrial aconitase activity. As a functional indicator of ROS production, aconitase activity underscores the protective effect of estrogen on mitochondrial enzymes. The difference in aconitase activity between 17β-estradiol and vehicle treatments is eliminated after enzyme reactivation with reducing reagents. This is consistent with a change in enzyme

activity, not protein expression, due to oxidation by mitochondrial ROS. Previously, using an *in vivo* hormone treatment paradigm, we showed physiological estrogen levels suppress brain mitochondrial oxidative stress (reflected in aconitase activity) in female and male rat brains (Razmara et al., 2007) and decrease hydrogen peroxide production in cerebrovascular mitochondria (Stirone et al., 2005b).

Estrogen can be an antioxidant through the quinol-product based redox cycle (Prokai et al., 2003). However, such direct antioxidant effects of estrogen require concentrations of at least 1–10 μM (Behl et al., 1997). In contrast, the neuroprotective effects of physiological estrogen levels that we and others report occur at much lower concentrations (1–100 nM) (Brinton et al., 2000; Wise, 2002). We show that 17α-estradiol, which does not activate ER but shares antioxidant activity (Manthey and Behl, 2006), does not mimic the effect of 17β-estradiol to decrease superoxide production. We also demonstrate that the action of 17β-estradiol is inhibited by an estrogen receptor antagonist. Together, these findings support the conclusion that the ability of estrogen to decrease superoxide production is not a direct antioxidant effect.

Of the two nuclear receptors for estrogen, this study demonstrates both ERα and ERβ in brain endothelial cells. Use of the selective agonists, PPT and DPN, showed that PPT (ERα selective) mimicked the effect of estrogen on superoxide production, but DPN (ERβ selective) had no effect. This supports the conclusion that estrogen acts via ERα to suppress mitochondrial oxidative stress.

Differential effects of SERMs on mitochondrial superoxide production highlight the selectivity and tissue-specific actions of these agents (Riggs and Hartmann, 2003). Raloxifene decreased superoxide production in HBMEC, while 4-hydroxy-tamoxifen had no effect by itself, but did block the action of estrogen. Both SERMs act in cerebral endothelial cells at concentrations that are within the range used clinically (Riggs and Hartmann, 2003). While raloxifene is used to treat osteoporosis, several studies indicate that it also has beneficial effects on the cardiovascular system (Vogelvang et al., 2006). These differential effects may provide insight in the development of SERMs as therapeutic agents to target brain endothelial mitochondria.

Although several mechanisms could mediate estrogen's effects on mitochondrial ROS production, we investigated MnSOD and cytochrome c. The mitochondrial form of SOD, MnSOD, is critical in superoxide metabolism. While transgenic homozygous MnSOD knockout mice are neonatal lethal due to acute superoxide toxicity (Li et al., 1995), even heterozygous MnSOD knockout mice demonstrate increased oxidative damage and age-related alterations in mitochondrial function (Kokoszka et al., 2001). We found no changes in mRNA, protein, or enzyme activity levels of MnSOD that could possibly explain the suppression of mitochondrial ROS after 24 h estrogen treatment. In contrast, we have shown previously that chronic estrogen treatment (three weeks) increases cerebrovascular mitochondrial MnSOD protein levels (Stirone et al., 2005b) and increases brain MnSOD activity (Razmara et al., 2007). Others have also reported this effect of estrogen (Krause et al., 2006). However, the scavenging of superoxide by MnSOD should lead to increased mitochondrial H_2O_2, which is then reduced to water by glutathione peroxidase-1 or catalase. In our previous study of *in vivo* estrogen exposure, mitochondrial H_2O_2 production was decreased, but without alterations of mitochondrial H_2O_2 scavenging enzymes, suggesting an overall decrease in ROS production, independent of MnSOD (Stirone et al., 2005b).

An alternative mechanism for the decrease in ROS production caused by estrogen concerns cytochrome c, a critical player in apoptosis and energy production. Cytochrome c has also recently been demonstrated to act as an antioxidant (Zhao et al., 2003) by shuttling electrons coming from superoxide anions, thereby decreasing mitochondrial ROS via an electron-leak pathway (Skulachev, 1998; Zhao and Xu, 2004). Previously, we have shown that *in vivo* and

in vitro estrogen treatment increases cerebrovascular mitochondrial cytochrome c levels (Stirone et al., 2005b). In HBMEC, we found that cytochrome c mRNA and protein levels were increased by estrogen, and siRNA experiments showed ERα to be responsible. These effects do not appear to be accounted for by an effect of estrogen to increase mitochondrial biogenesis, because cytochrome c protein measurements were corrected for by levels of the mitochondrial membrane protein porin. It is possible that estrogen enhances mitochondrial efficiency while suppressing superoxide production by changing levels of cytochrome c. Thus, the ERα mediated increase in cytochrome c may not only improve the efficiency of the respiratory chain, but also reduce mitochondrial ROS through the electron-leak pathway.

Estrogen may also decrease mitochondrial ROS by other mechanisms. Mitochondrial superoxide production can be affected by uncoupling proteins, but we recently demonstrated that estrogen has no effect on these proteins (Razmara et al., 2007). Estrogen may also influence the interaction between nitric oxide (NO) and mitochondrial ROS production in endothelial cells (Moncada and Higgs, 2006). Previously, we showed that estrogen exposure increases expression and phosphorylation of endothelial nitric oxide synthase, thus increasing NO production (Stirone et al., 2005a). NO can scavenge superoxide anions directly via formation of peroxynitrite species or indirectly via cytochrome c stabilization (Zhang and Gutterman, 2007). These mechanisms may also contribute to mitochondrial effects of estrogen.

In summary, we demonstrate a profound suppressive effect of estrogen on brain endothelial mitochondrial ROS production. While estrogen may modulate mitochondrial oxidative stress through several independent mechanisms, the concomitant increase in cytochrome c may be a major factor. Nevertheless, other potential pathways need to be explored. With clear evidence that blood-brain barrier disruption occurs early in the development of many neurological pathologies (Abbott et al., 2006), the cerebral vasculature has received increasing focus as a possible therapeutic target. Maintaining brain endothelial health and function, by protecting mitochondria from accumulation of ROS damage, may potentially delay the onset of age-related neurodegenerative diseases. The current study identifies a profound effect of estrogen on brain endothelial mitochondrial oxidative stress as another potential novel pathway to protect function of the cerebral circulation. Selective estrogen receptor modulators that target cerebral endothelial mitochondria may protect against stroke and delay development of age-related neurodegenerative diseases.

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List of non-standard abbreviations

ER
: estrogen receptors

HBMEC
: human brain microvascular endothelial cells

MnSOD
: Mn superoxide dismutase

mtDNA
: mitochondrial DNA

NO
nitric oxide

RNAi
RNA interference

ROS
reactive oxygen species

SERM
selective estrogen receptor modulator

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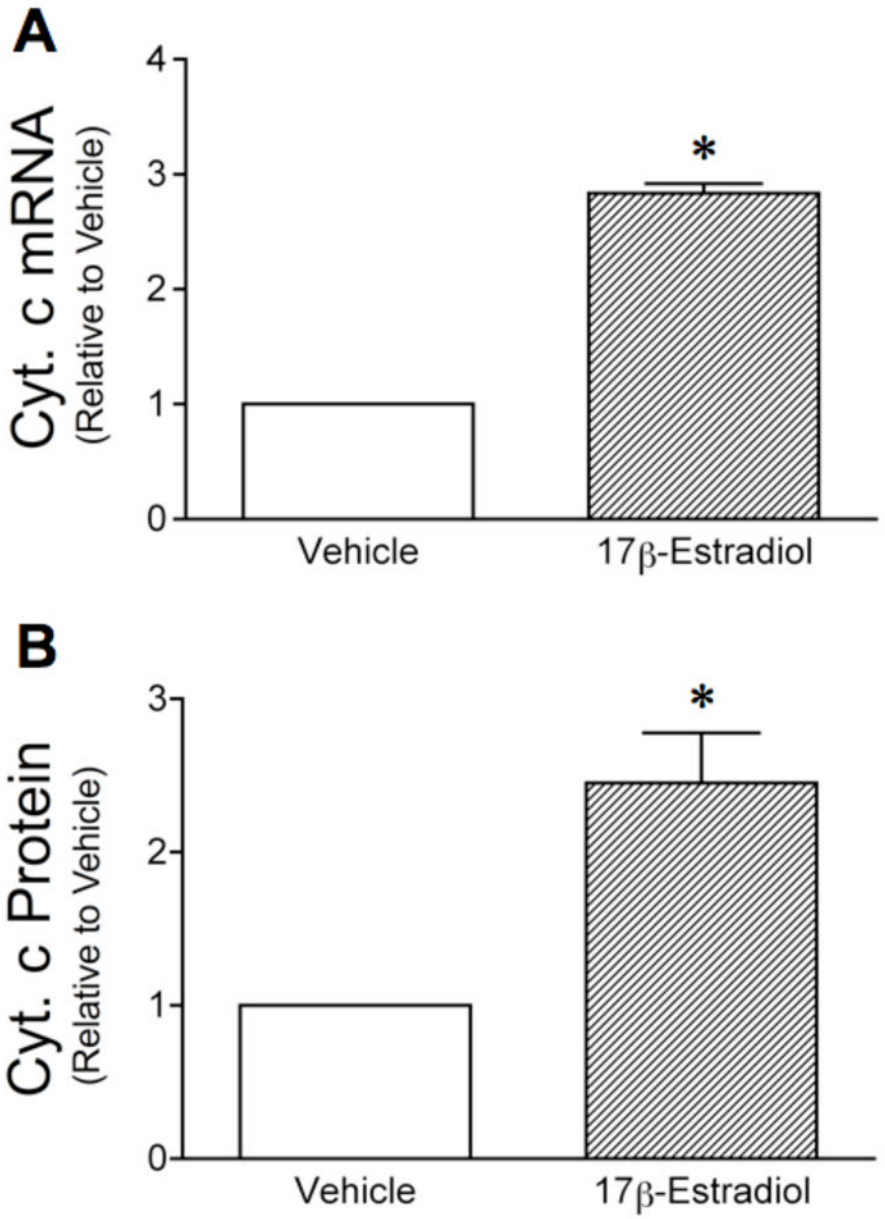

Fig. 1.

Effect of estrogen on cytochrome c. After 24 h treatment with 17β-estradiol (10 nM) and vehicle control, cytochrome c mRNA and protein were measured. A. Quantitative real-time PCR measurement for cytochrome c mRNA. Data were normalized to beta-actin as an internal control and expressed relative to vehicle control. Values are means ± SEM; *Significantly different than vehicle; $P \leq 0.05$; n=4. B. Mean cytochrome c protein levels in estrogen-treated HBMEC and vehicle control and expressed relative to vehicle control. Values are means ± SEM; *Significantly different than vehicle; $P \leq 0.05$; n=4.

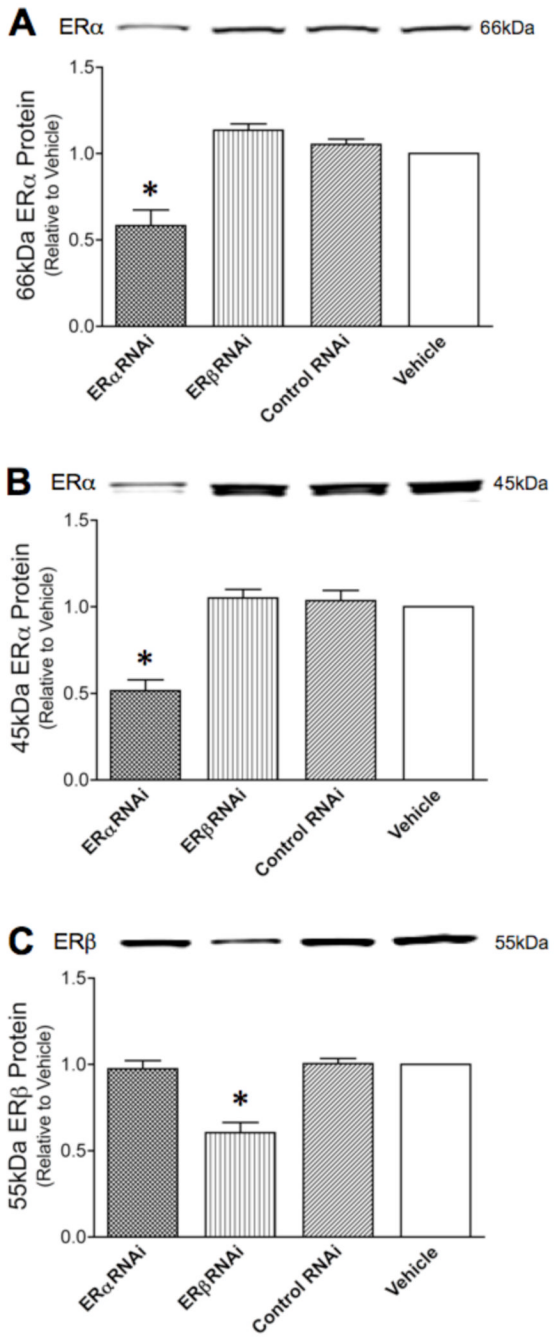

Fig. 2.

Effect of estrogen receptor RNA interference on levels of ERα and ERβ in HBMEC after 48 h transfection with either ERα RNAi, ERβ RNAi, a negative control RNAi construct or vehicle. Immunoblots show bands corresponding to the 66 kDa (A) and 45 kDa (B) forms of ERα and ERβ (C). Following densitometric analysis of each blot, all values were expressed relative to the vehicle-treated cells; means ± SEM are shown. *Significantly different than vehicle; $P \leq 0.05$; n=4.

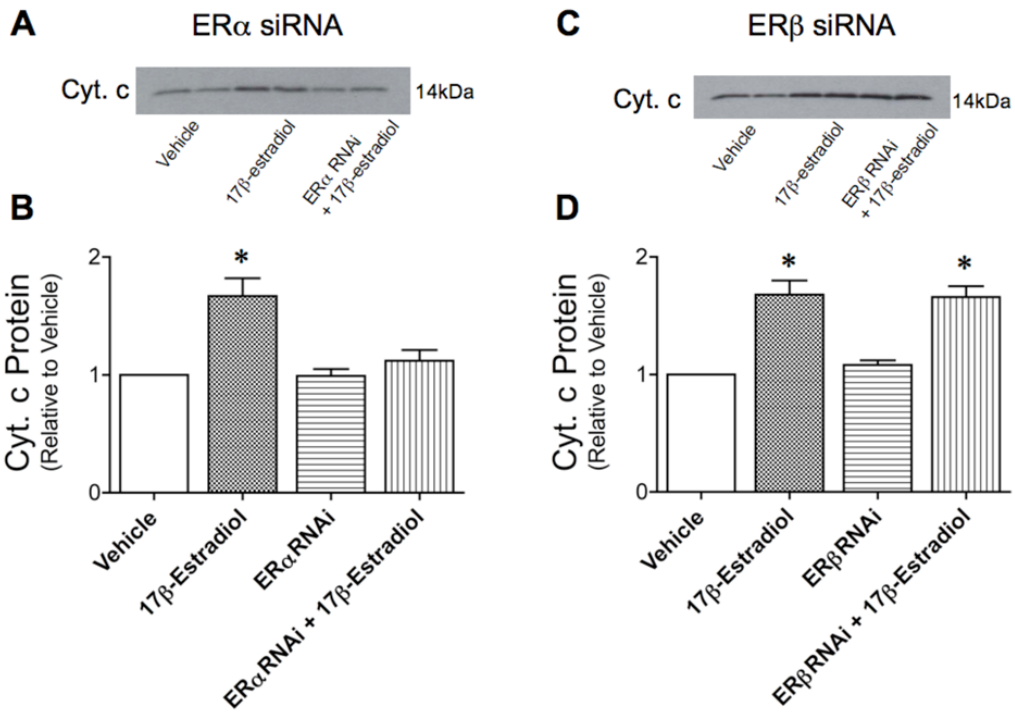

Fig. 3.

Effect of estrogen treatment and ER RNA interference on levels of cytochrome c. After 48 h transfection with either ERα or ERβ RNAi construct or vehicle treatment, HBMEC were then treated with 10 nM 17β-estradiol for 24 h. Representative Western blot (A) and densitometric analysis (B) comparing cytochrome c protein levels in HBMEC treated with 17β-estradiol in the presence or absence of RNAi for ERα and expressed relative to vehicle control. Values are means ± SEM; *Significantly different than vehicle; $P \leq 0.05$; n=4. Representative immunoblot (C) and densitometric analysis (D) for levels of cytochrome c after treatment with 17β-estradiol in presence or absence of RNAi for ERβ and expressed relative to vehicle control. Values are means ± SEM; *Significantly different than vehicle; $P \leq 0.05$; n=4.

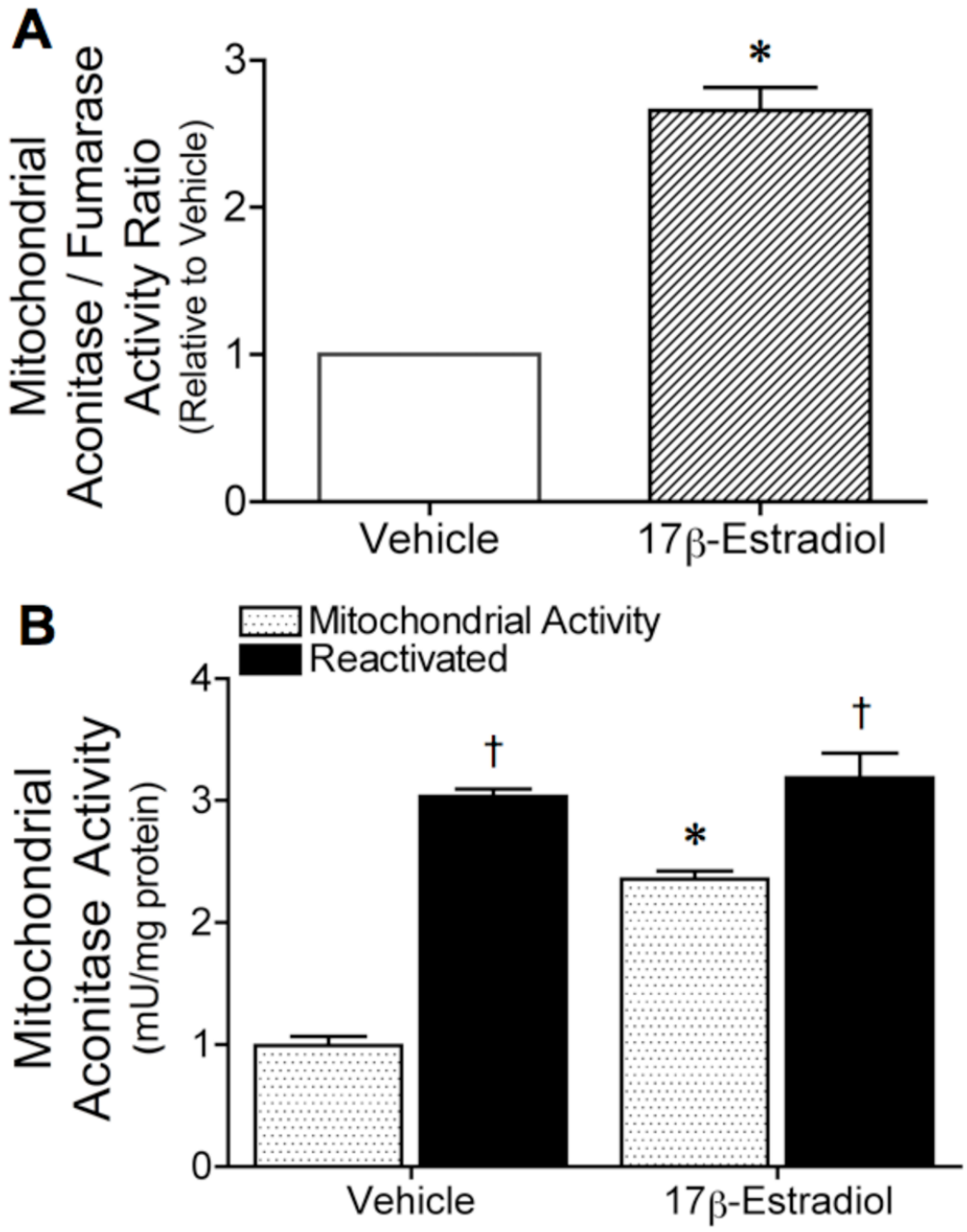

Fig. 4.

Effects of estrogen treatment on mitochondrial aconitase activity. Mitochondria were isolated from 17β-estradiol-treated and vehicle-treated control HBMEC. A. The ratio of activities of aconitase, inactivated by ROS, to fumarase, unaffected by ROS, was measured as a functional indicator of mitochondrial ROS production. The activity ratio relative to vehicle control is shown. Values are means ± SEM. *Significantly different from vehicle control; $P \leq 0.05$; n=4. B. Aconitase activity measured in isolated HBMEC mitochondria before and after total enzyme reactivation with reducing reagents. Values are means ± SEM. *Significantly different from mitochondrial activity of vehicle control group; $P \leq 0.05$; n=4. †Significantly different from activity before reactivation within each group; $P \leq 0.05$; n=4.

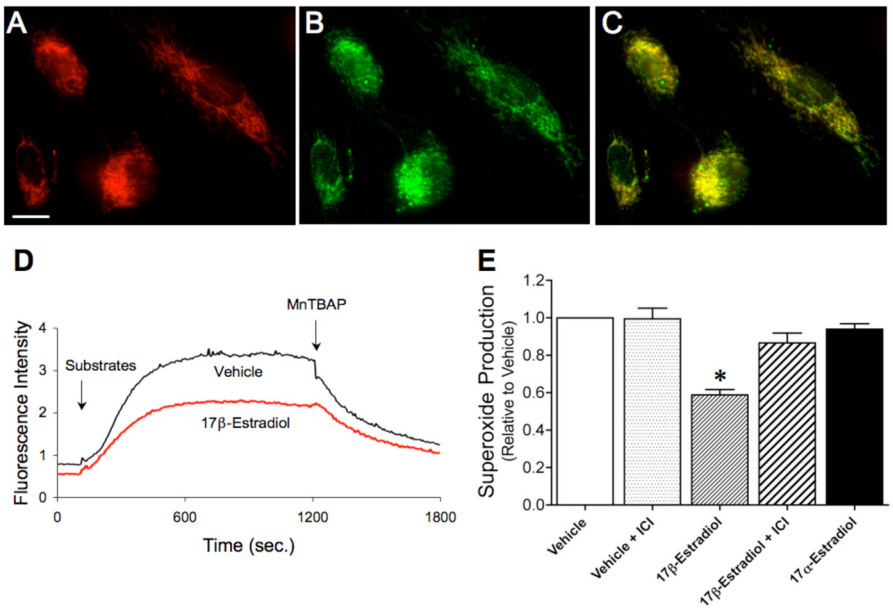

Fig. 5.

Effects of estrogen on mitochondrial superoxide production. HBMEC were treated with 10 nM 17β-estradiol, 10 nM 17α-estradiol or vehicle control. Some HBMEC were pre-equilibrated with the estrogen receptor antagonist ICI-182,780 (1 μM) for 1 h and maintained during 17β-estradiol or vehicle treatment. Cells were exposed to these treatments for 24 h prior to measurement of superoxide production in live cells using the MitoSOX Red dye. A. MitoSOX Red mitochondrial superoxide indicator staining shown in red. Scale bar, 10 μm. B. Subunit I of complex IV, a mitochondrial DNA encoded-protein, staining shown in green. C. Immunofluorescence co-localization (yellow) of MitoSOX Red and subunit I of complex IV. D. Representative tracing of MitoSOX dye fluorescence intensity reflecting mitochondrial superoxide levels. Tracings for HBMEC pre-treated with 17β-estradiol or vehicle are shown. Pyruvate and malate, complex I substrates, both at 2 mM, were added to initiate the reaction. MnTBAP, a superoxide dismutase mimetic, was added after a plateau was reached. E. Mean values of mitochondrial superoxide production, corrected for sample protein concentration and expressed relative to vehicle control are shown. Values are means ± SEM. *Significantly different from other groups; $P \leq 0.05$; n=8 for vehicle and 17β-estradiol groups; n=4 for other groups.

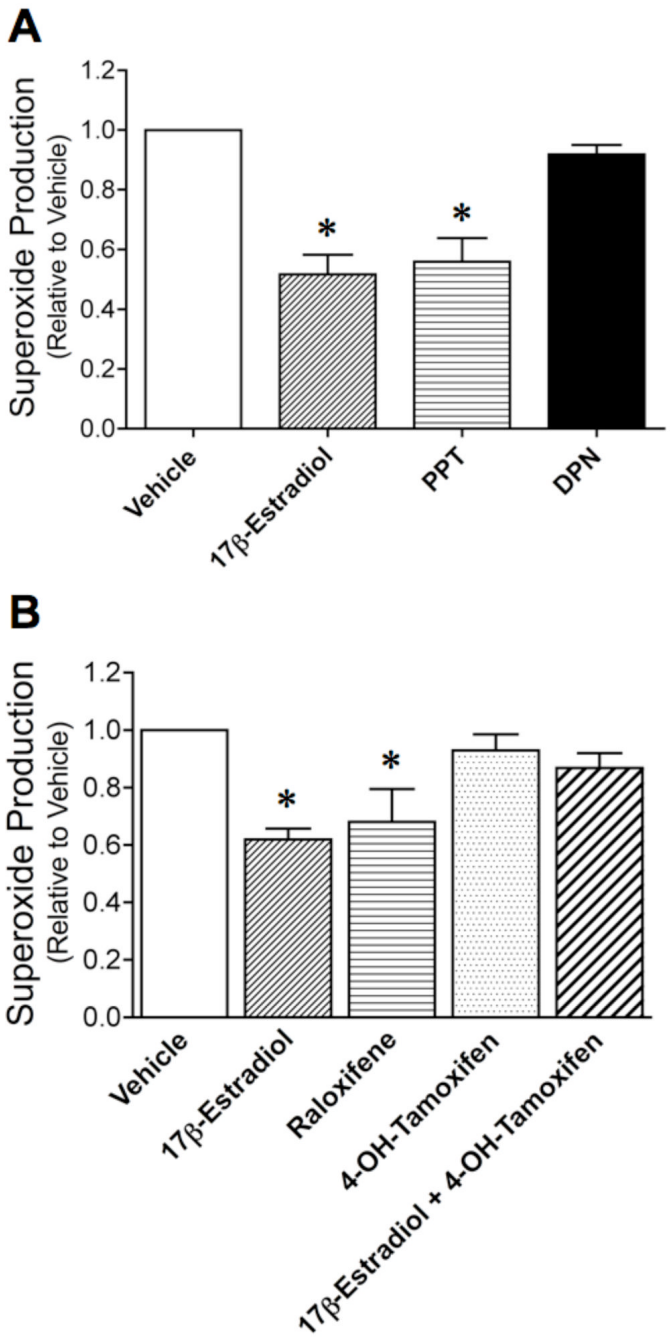

Fig. 6.

Effects of selective ER agonists and ER modulators on mitochondrial superoxide production. HBMEC were treated with 10 nM 17β-estradiol, 10 nM 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT), 10 nM 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN), 100 nM 4-hydroxy-tamoxifen, 100 nM raloxifene, or vehicle control. Some HBMEC were pre-equilibrated with 100 nM 4-hydroxy-tamoxifen for 1 h and maintained during 17β-estradiol treatment. A. The effects of the selective ER agonists, PPT and DPN, on the production of mitochondrial superoxide. B. The effects of the selective ER modulators, raloxifene and 4-hydroxy-tamoxifen (4-OH-tamoxifen), on mitochondrial superoxide production. Mean values of mitochondrial superoxide production, corrected for sample protein concentration and

expressed relative to vehicle control are shown. Values are means ± SEM. *Significantly different from all groups without asterisk; $P \leq 0.05$; n=8 for vehicle and 17β-estradiol groups; n=4 for all other groups.

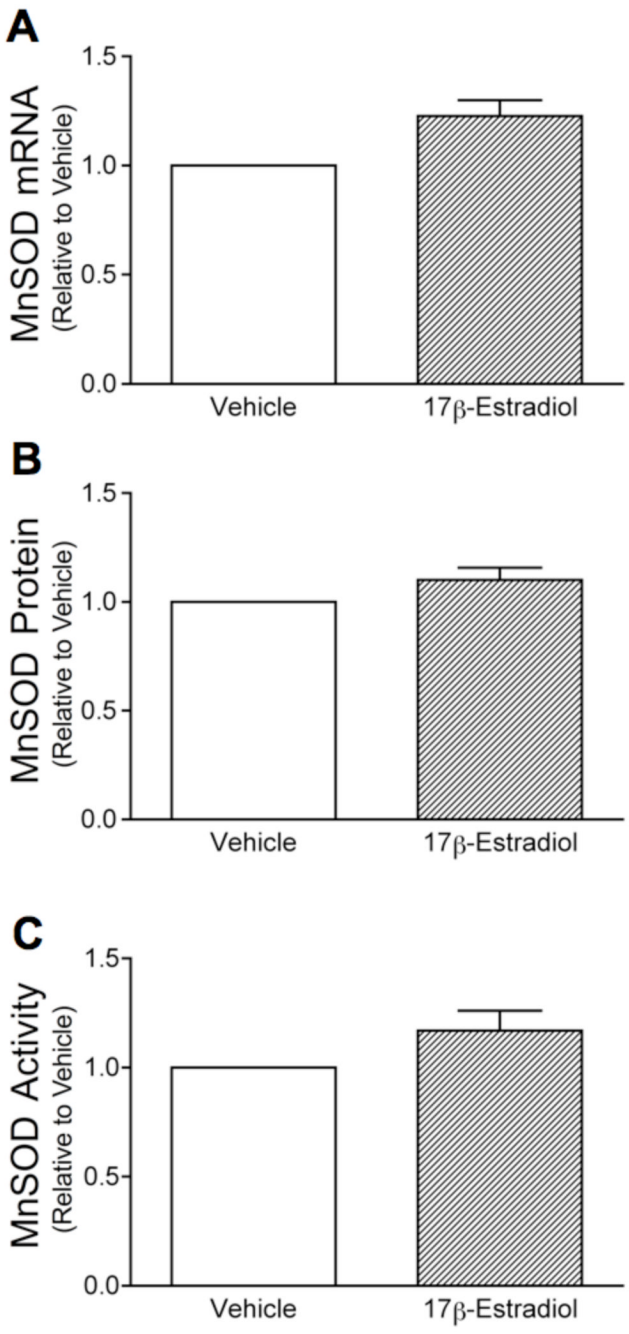

Fig. 7.

Effects of estrogen on manganese superoxide dismutase (MnSOD). After 24 h treatment with estrogen and vehicle control, MnSOD mRNA, protein, and enzyme activity were measured. A. Quantitative real-time PCR measurement for MnSOD mRNA. Data were normalized to beta-actin as an internal control and expressed relative to vehicle control. Values are means ± SEM; $P > 0.05$; n=4. B. Mean MnSOD protein levels in mitochondria isolated from 17β-estradiol-treated and vehicle-treated control HBMEC and expressed relative to vehicle control. Values are means ± SEM; $P > 0.05$; n=4. C. Activity of MnSOD in mitochondria isolated from HBMEC treated with either 10 nM 17β-estradiol or vehicle control for 24 h expressed relative to vehicle control. Values are means ± SEM; $P > 0.05$; n=6.